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Sazetidine-A Is a Potent and Selective Agonist at Native and Recombinant $\alpha4\beta2$ Nicotinic Acetylcholine Receptors

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ABSTRACT

Sazetidine-A has been recently proposed to be a "silent desensitizer" of $\alpha4\beta2$ nicotinic acetylcholine receptors (nAChRs), implying that it desensitizes $\alpha4\beta2$ nAChRs without first activating them. This unusual pharmacological property of sazetidine-A makes it, potentially, an excellent research tool to distinguish between the role of activation and desensitization of $\alpha4\beta2$ nAChRs in mediating the central nervous system effects of nicotine itself, as well as those of new nicotinic drugs. We were surprised to find that sazetidine-A potently and efficaciously stimulated nAChR-mediated dopamine release from rat striatal slices, which is mediated by $\alpha4\beta2^*$ and $\alpha6\beta2^*$ subtypes of nAChR. The agonist effects on native striatal nAChRs prompted us to re-examine the effects of sazetidine-A on recombinant $\alpha4\beta2$ nAChRs in more detail. We expressed the two alternative stoichiometries of $\alpha4\beta2$ nAChR in *Xenopus laevis* oocytes and investigated the agonist properties of sazetidine-A on both $\alpha4(2)\beta2(3)$and $\alpha4(3)\beta2(2)$ nAChRs. We found that sazetidine-A potently activated both stoichiometries of $\alpha4\beta2$ nAChR: it was a full agonist on $\alpha4(2)\beta2(3)$ nAChRs, whereas it had an efficacy of only 6% on $\alpha4(3)\beta2(2)$ nAChRs. In contrast to what has been published before, we therefore conclude that sazetidine-A is an agonist of native and recombinant $\alpha4\beta2$ nAChRs but shows differential efficacy on $\alpha4\beta2$ nAChRs subtypes.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels, expressed throughout the central and peripheral nervous system. Molecular cloning has provided evidence for the existence of at least nine different types of nAChR α subunits ($\alpha2$-$\alpha10$) and three different types of nAChR β subunits ($\beta2$-$\beta4$), in the nervous system. Some of these subunits form homopentameric receptors when expressed in heterologous expression systems ($\alpha7$, $\alpha8$, and $\alpha9$), whereas other subunits assemble into heteropentameric structures with various combinations of α and β subunits. Different subunit combinations yield functional nAChRs that differ considerably in their functional and pharmacological properties (Luetje and Patrick, 1991; Papke, 1993; Chavez-Noriega et al., 1997). The predominant subtype of nAChR in the central nervous system contains $\alpha4$ and $\beta2$ subunits ($\alpha4\beta2^*$) (Flores et al., 1992; Picciotto et al., 2001). The asterisk indicates that besides $\alpha4$ and $\beta2$ subunits, there may be additional subunits coassembled in native $\alpha4\beta2$ receptors. These $\alpha4\beta2^*$ nAChRs are possible targets for drugs to treat pain, nicotine addiction, attention deficit disorders, and such diseases as Alzheimer's and Parkinson's (Lloyd and Williams, 2000; Jensen et al., 2005). The exact subunit composition of $\alpha4\beta2^*$ nAChRs in the brain is still largely unknown.

Epibatidine, a chemical originally isolated from the skin of the Ecuadorian poison-arrow frog, *Epipedobates tricolor*, has been shown to act as a nAChR agonist and to cause profound analgesic effects (Badio and Daly, 1994). However, apart from showing strong analgesic effects, epibatidine also causes a wide range of severe side effects, possibly because of its lack of subtype specificity, which makes it far too toxic to be considered for clinical use (Rupniak et al., 1994; Sullivan et al., 1994a,b; Boyce et al., 2000). In the last few years, significant efforts have been directed toward the discovery of new nAChR-based analgesics with improved safety profiles. One focus has been on selectivity [i.e., trying to identify nAChR agonists that selectively activate those nAChR subtypes that are involved in causing the positive analgesic effect (mainly $\alpha4\beta2^*$) but don't activate those nAChRs (e.g., $\alpha3\beta4$, $\alpha1\beta1\gamma\varepsilon\delta$) that are involved in mediating the most severe side effects (Ji et al., 2007)]. In addition, variations in potency and efficacy of selective $\alpha4\beta2$ nAChR agonists have

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine.

been explored. Along these lines, sazetidine-A has been reported to bind selectively and with high affinity to $\alpha4\beta2$ nAChRs (Xiao et al., 2006). The reported functional properties of this compound, however, are uncommon. It has been shown that sazetidine-A can potently desensitize $\alpha4\beta2$ nAChRs without activating them. It has been suggested that sazetidine-A could represent a new class of nicotinic cholinergic drugs with a novel mechanism of action, branded as "silent desensitizers." On the other hand, recent reports suggest in vivo efficacy strikingly similar to more conventional $\alpha4\beta2$ agonists. For example, in rats that were trained to discriminate nicotine from saline, sazetidine-A was demonstrated to fully substitute for nicotine as discriminative stimulus in a drug discrimination assay (Xiao et al., 2007). Furthermore, sazetidine-A was found to be more potent and efficacious than epibatidine in the formalin test for persistent pain (Cucchiaro et al., 2007). Because of the intriguing discrepancy between the in vitro and in vivo reports, we decided to characterize sazetidine's in vitro pharmacological properties in more detail. We were surprised to find that sazetidine-A was a potent agonist on native $\alpha4\beta2$* nAChRs mediating dopamine release from rat striatal slices. Further in vitro characterization of the compound on distinct human recombinant $\alpha4\beta2$ nAChR stoichiometries revealed that the receptors with the $\alpha4(2)\beta2(3)$ stoichiometry, are also potently and efficaciously activated by sazetidine-A. Our results, showing that sazetidine-A is a potent agonist at native and recombinant $\alpha4\beta2$ nAChRs, reconcile the apparent discrepancy between in vitro and in vivo results previously reported.

Materials and Methods

Sazetidine-A

Sazetidine-A (Fig. 1) was synthesized as described previously (Xiao et al., 2006) with modification on the step of Sonogashira coupling reaction. Thus, a mixture of (*S*)-5-bromo-3-{[1-(*t*-butoxycarbonyl)-2-azetinyl]-methoxy}pyridine (1.213 g, 3.53 mmol), potassium carbonate (1.22 g, 8.84 mmol), triphenylphosphine (148 mg, 0.57 mmol), copper(I) bromide (81 mg, 0.57 mmol), and palladium on carbon (100 mg) in 1,2-dimethoxyethane (14 ml) and water (14 ml) was stirred at room temperature for 30 min under N_2, then 5-hexyn-1-ol (867 mg, 8.84 mmol) was added to the mixture. The reaction was heated to reflux for 68 h. The reaction was diluted with EtOAc, the organic layer was separated, and the aqueous layer was extracted with EtOAc twice. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by chromatography (silica gel) eluting with EtOAc/hexanes 50 to 80% to afford (*S*)-5-(5-hexyn-1-ol)-3-{[1-*t*-(butoxycarbonyl)-2-azetidinyl]-methoxy}pyridine as a colorless oil (1.162 g, 91.2%). ^{1}H NMR data match the reported data on this compound. Deprotection of (*S*)-5-(5-hexyn-1-ol)-3-{[1-*t*-(butoxycarbonyl)-2-azetidinyl]-methoxy}pyridine provided Sazetidine-A. The purity of the final product is 100% as measured by liquid chromatography-mass spectrometry. An alternative batch of sazetidine-A was purchased from Alexis Biochemicals (Nottingham, UK).

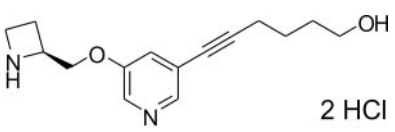

Fig. 1. Chemical structure of sazetidine-A.

[^{3}H]Epibatidine Binding to $\alpha4\beta2$ and $\alpha3\beta4$ nAChRs Stably Expressed in HEK-293 Cells

Membrane Preparation. HEK293 cell lines stably expressing the nicotinic $\alpha4\beta2$ or $\alpha3\beta4$ receptors were maintained in Dulbecco's modified Eagle's medium/Nutrient Mix F-12 (3:1; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), 250 μg/ml G418 (Geneticin), and 20 mM HEPES (Invitrogen). Membranes from large-scale cell production were prepared as described previously (Smith et al., 2007). In brief, cell pastes were homogenized in 4 volumes of buffer (50 mM Tris-HCl, 150 mM NaCl, and 5 mM KCl, pH 7.4). The homogenate was centrifuged twice (40,000*g*, 10 min, 4°C), and the pellets were resuspended in 4 volumes of Tris-HCl buffer after the first spin and 8 volumes after the second spin. The resuspended homogenate and the supernatant were centrifuged again (100*g*, 10 min, 4°C and 40,000*g*, 20 min, 4°C, respectively), and the pellet was resuspended in Tris-HCl buffer supplemented with 10% (w/v) sucrose. The membrane preparation was stored in 1-ml aliquots at −80°C until tested. The protein concentration of the membrane preparation was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL).

Nicotinic Receptor Radioligand Binding Scintillation Proximity Assay. Scintillation proximity assay radioligand binding assays were modified from methods described previously (Badio and Daly, 1994; Gerzanich et al., 1995). Both $\alpha4\beta2$ and $\alpha3\beta4$ assays were performed in 96-well plates in a final volume of 250 μl of Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl, and 5 mM KCl, pH 7.4) using the following conditions: 2 nM [^{3}H]epibatidine (53 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK); 1 mg/well wheat germ agglutinin-scintillation proximity assay beads (GE Healthcare); and 5 μg/well of membrane protein. Nonspecific binding (<10% for both assay types) was determined using 100 μM concentrations of unlabeled epibatidine. Reactions were allowed to equilibrate for 3 h at room temperature before reading on a Wallac counter (PerkinElmer Life and Analytical Sciences). Data were analyzed as K_i values using a four-parameter logistic curve fitting program (ActivityBase v5.3.1.22l IDBS, Guildford, Surrey, UK).

[^{3}H]Dopamine and [^{3}H]Noradrenaline Release from Rat Native Tissue

Male Lister hooded rats (250–350 g) were killed by exposure to CO_2 followed by cervical dislocation. Striata from two rats or hippocampi from three rats were dissected and chopped three times at 150 μm using a McIlwain tissue chopper, each time rotating the tissue through 60°. Slices were dispersed in Krebs bicarbonate buffer (118 mM NaCl, 2.4 mM KCl, 2.4 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 10 mM glucose, 1 mM ascorbic acid, gassed with 5% CO_2/95% O_2 for 1 h, pH 7.4), including 10 μM pargyline, and were incubated with [^{3}H]dopamine (20–23 μCi/ml; 50 nM) or [^{3}H]noradrenaline (56–60 μCi/ml; 100 nM) for 30 min at 37°C. After loading, slices were washed with Krebs buffer containing 1 μM nomifensine and 10 μM pargyline. After the final wash, slices were resuspended in Krebs buffer, and a 100-μl aliquot was placed in each well of a 96-well GF/C filter plate (Millipore, Billerica, MA). Buffer was removed to waste, and 70 μl of buffer was added to each well before incubation at 37°C for 5 min, after which the buffer was removed into a 96-well collection plate. Slices were then stimulated for 5 min with agonist (70 μl/well), after which the stimulating buffer was removed into another 96-well collection plate. Optiphase Supermix (PerkinElmer Life and Analytical Sciences) scintillation fluid (170 μl) was added to each well of the collection plates before the plates were heat-sealed and radioactivity was quantified using a 1450 Microbeta 96-well plate counter (counting efficiency, 25%; PerkinElmer Life and Analytical Sciences). Radioactivity remaining in the slices was measured by digestion of the tissue in 1 M HCl for 1 h with release quantified as above. Release of [^{3}H]dopamine or [^{3}H]noradrenaline was expressed as a fraction of the total radioactivity contained within the slices at the time of stimulation. Data points are shown as mean ± S.E.M. of at least

three independent experiments (each with four or more replicates). Curves were fitted using the four-parameter Hill equation using Sigmaplot 9.0. α-Conotoxin MII was purchased from Tocris Bioscience (Bristol, UK); dihydro-β-erythroidine and mecamylamine were purchased from Sigma-Aldrich (Poole, UK).

X. laevis Oocyte Expression and Electrophysiological Recordings

Stage V and VI *X. laevis* oocytes were prepared using standard procedures (Chavez-Noriega et al., 1997). Human α4 and β2 subunit cDNAs, ligated into the pCI (Promega) expression vector, were dissolved in distilled water at a concentration of 1 μg/μl (spectrophotometric and agarose gel electrophoresis determinations). Mixtures of α4 and β2 cDNA at 1:10, 10:1 ratios were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). The total amount of cDNA injected per oocyte was kept constant at 2 ng. After injection, oocytes were incubated at 18°C for 2 to 5 days in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 5 mg/l neomycin, pH 7.6. Recordings were performed 3 to 5 days after injection. Oocytes were placed in a 0.1-ml recording chamber and perfused with modified Ringer solution (150 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 1.8 mM $BaCl_2$, pH 7.2, adjusted with NaOH) at a rate of 10 ml/min. We chose a nominally Ca^{2+}-free solution to minimize the contribution to the response of Ca^{2+}-gated chloride channels that are endogenous to the *X. laevis* oocyte and may be activated by Ca^{2+} entry through the nAChRs.

Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl (0.5–2.0 MΩ) and voltage-clamped at −60 mV using a GeneClamp 500B amplifier and pClamp 6 software (Molecular Devices, Sunnyvale, CA). Typically, traces were filtered at 1 kHz during recording and digitized at 0.5 - 5kHz using the DigiData 1200 interface (Axon Instruments, CA). All experiments were carried out at room temperature. Agonist concentration-response curves were obtained by normalizing agonist-induced responses to the control responses induced by 1 mM ACh (a near-maximum effective concentration at receptors obtained with 10:1 α4/β2 cDNA transfecting ratios and an EC_{100} concentration at receptors expressed by oocytes injected with 1:10 α4/β2 cDNA ratios). A minimum interval of 4 min was allowed between agonist applications, as this was found to be sufficient to ensure reproducible recordings.

Data Analysis

Concentration-response curves were fitted by a nonlinear least-squares algorithm according to the equation $i = i_{max} / (1 + \{EC_{50}/[conc]\}^n)$, in which i_{max} is the maximum obtainable peak current, EC_{50} is the concentration of the agonist that elicits 50% of the maximum obtainable peak current, and n is the slope factor. Results are expressed as mean ± S.D.

Results

Radioligand Binding Assays. The binding selectivity of sazetidine-A for human recombinant α4β2 versus human recombinant α3β4 nAChRs was evaluated in competition experiments using [^{3}H]epibatidine as the radioligand. Using standard saturation binding methods, the K_d values of epibatidine for α4β2 and α3β4 membranes were determined to be 0.24 and 0.37 nM, respectively. Sazetidine-A displaced [^{3}H]epibatidine binding to α4β2 with a mean relative IC_{50} of 2.6 ± 1.2 nM, a calculated mean K_i of 0.26 ± 0.11 nM, and a mean Hill coefficient of 0.85 ± 0.03. On the other hand, sazetidine-A displaced [^{3}H]epibatidine binding to α3β4 with a mean relative IC_{50} of 365 ± 59 nM, a calculated mean K_i of 54 ± 5 nM, and a mean Hill coefficient of 0.85 ± 0.11 (Fig. 2). We therefore confirmed previously published binding results (Xiao et al., 2006) showing that sazetidine-A binds with significantly higher affinity to α4β2 than α3β4, and extended this finding to human receptors. It is worth noting, however, that the selectivity ratios between human receptors (current study) seem to be smaller than that for rat receptors (Xiao et al., 2006). Species specificity issues, specifically between human and rat α3β4 nAChRs, have recently been highlighted by our laboratory (Young et al., 2007).

Neurotransmitter Release Assays. Binding assays per se cannot predict whether a ligand acts as an agonist or an antagonist at a particular receptor. Functional assays with sazetidine-A on native rat nAChRs have therefore been performed to answer this specific question. Dopamine release from rat striatal slices is mediated by the activation of native α4β2* and α6β2* nAChRs (Mogg et al., 2004; Salminen et al., 2004; Grady et al., 2007; Smith et al., 2007). We found that sazetidine-A acts as a potent and efficacious agonist in evoking [^{3}H]dopamine release from rat striatal slices (EC_{50} = 1.1 ± 0.3 nM; E_{max} = 96 ± 6%; n = 3; Fig. 3a). Both the nicotinic antagonists mecamylamine (n = 3) and dihydro-β-erythroidine (n = 3) inhibited sazetidine-A-evoked dopamine release, demonstrating the nicotinic nature of the sazetidine-A-evoked response. α-Conotoxin MII is a nicotinic antagonist that distinguishes between α4β2* and α6β2* nAChRs. α4β2* nAChRs are resistant to 100 nM toxin, whereas α6β2* nAChRs are fully blocked by the same toxin concentration (Salminen et al., 2004). At the concentration of 100 nM α-conotoxin MII reduced the E_{max} but had no significant effect on the EC_{50} of sazetidine-A. The estimates of EC_{50} and E_{max} values in the presence of 100 nM α-contotoxin MII are 0.27 ± 0.5 nM and 49 ± 1% (n = 3), respectively, indicating that 52% of the sazetidine-A induced DA release is mediated by α-conotoxin MII-resistant (α4β2*) nAChRs and that 48% of the sazetidine-A-induced DA release is mediated by α-conotoxin MII-sensitive (α6β2*) nAChRs.

Noradrenaline release from rat hippocampal slices is mediated by the activation of native α3β4* nAChRs (Luo et al., 1998; Anderson et al., 2000). We found that sazetidine-A also evoked noradrenaline release but with much lower potency and lower efficacy (EC_{50} = 4.5 ± 2.6 μM, and E_{max} = 55 ± 7%; n = 3) than it evoked dopamine release from striatal slices. The nicotinic antagonists mecamylamine and dihydro-β-erythroidine both in-

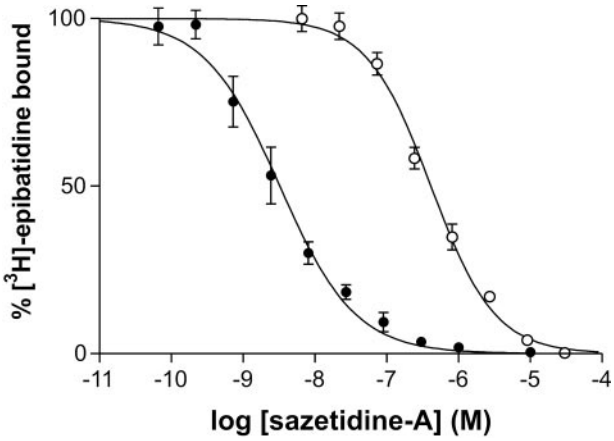

Fig. 2. Receptor binding experiments. Sazetidine-A displaces the radioligand [3H]epibatidine from its binding sites on α4β2 nAChRs (●) and α3β4 nAChRs (○). Data are expressed as mean ± S.E.M. for three experiments.

hibited sazetidine-A-evoked [^{3}H]dopamine release (Fig. 3b), confirming that sazetidine-A effects are mediated by nAChR activation, rather than by other means. These neurotransmitter release experiments from native rat tissues demonstrate that sazetidine-A is a potent agonist of native rat $\alpha4\beta2$* and $\alpha6\beta2$* receptors but a weak agonist of native $\alpha3\beta4$* nAChRs.

To verify that the agonist effects of sazetidine-A observed are not caused by the specific batch of sazetidine-A used, we also tested another, commercially available sazetidine-A. The EC_{50} and E_{max} values obtained with this new source of sazetidine-A were 0.85 ± 0.28 nM and 94 ± 3% (n = 3), respectively, in the dopamine release assay from striatal slices and 24 ± 6 μM and 38 ± 3% (n = 3), respectively, in the noradrenaline release assay from hippocampal slices (data not shown). These data are consistent with the data obtained with the sazetidine-A that we synthesized in house.

Oocyte Expression Experiments. Based on the intriguing new finding described above, we decided to revisit the functional properties of sazetidine-A with the aim of 1) confirming that the agonist effects on $\alpha4\beta2$* nAChRs we saw were not specific to the rat but also translate to the human receptors and 2) understanding better the biophysical basis for the absence of agonism previously reported (Xiao et al., 2006). We chose to use the *X. laevis* oocyte expression system, combined with the two-electrode voltage clamp technique, because this is a very sensitive technique that can also detect very partial agonism. In addition, the oocyte expression system can be used to study different subunit stoichiometries of $\alpha4\beta2$ nAChR simply by varying the $\alpha4$ and $\beta2$ subunit cDNA ratio that is injected before the experiments. $\alpha4(2)\beta2(3)$ and $\alpha4(3)\beta2(2)$ nAChRs can then be studied in isolation and, importantly, have previously been shown to have distinct pharmacology (Moroni et al., 2006; Zwart et al., 2006). Figure 4a shows the concentration-dependence of the peak amplitudes of currents induced by sazetidine-A on both subtypes of $\alpha4\beta2$ nAChR. Various concentrations of sazetidine-A and the maximally effective concentration of 1 mM ACh (Moroni et al., 2006) were alternately applied to control for rundown of the response. Sazetidine-A-induced current amplitudes were normalized to the amplitude of 1 mM ACh-induced currents. Mean concentration-response curves calculated from the parameters obtained by fitting the equation under *Data Analysis* to the data of individual experiments are depicted in Fig. 4a. The mean EC_{50}, slope factor, and E_{max} of the concentration-response curves for sazetidine-A (n = 3) are 6.1 ± 1.2 nM, 1.0 ± 0.1, and 98 ± 9%, respectively, for $\alpha4(2)\beta2(3)$ nAChRs and 2.4 ± 1.2 nM, 1.3 ± 0.4, and 5.8 ± 1.1%, respectively, for $\alpha4(3)\beta2(2)$ nAChRs. Figure 4b shows examples of currents induced by 1 mM ACh and 100 nM sazetidine-A, which are maximally effective concentrations for the two agonists at $\alpha4(2)\beta2(3)$ nAChRs. Sazetidine-A was clearly able to evoke currents of approximately the same size as those induced by ACh. On the other hand, Fig. 4c shows examples of currents induced by 1 mM ACh and 1 μM sazetidine-A, which are maximally effective concentrations for the two agonists at $\alpha4(3)\beta2(2)$ nAChRs. These traces highlight that sazetidine-A is a very poorly efficacious agonist at $\alpha4(3)\beta2(2)$ nAChRs.

Discussion

The most striking finding of this investigation is that, contrary to previous reports (Xiao et al., 2006), sazetidine-A is a potent and efficacious agonist of both human recombinant and rat native $\alpha4\beta2$* nAChRs.

Addition of sazetidine-A to rat striatal slices potently and efficaciously stimulated dopamine release, an effect blocked by the nicotinic antagonists dihydro-β-erythroidine and mecamylamine. It is noteworthy that 100 nM α-conotoxin MII, a nicotinic antagonist that distinguishes $\alpha4\beta2$* nAChRs from $\alpha6\beta2$*

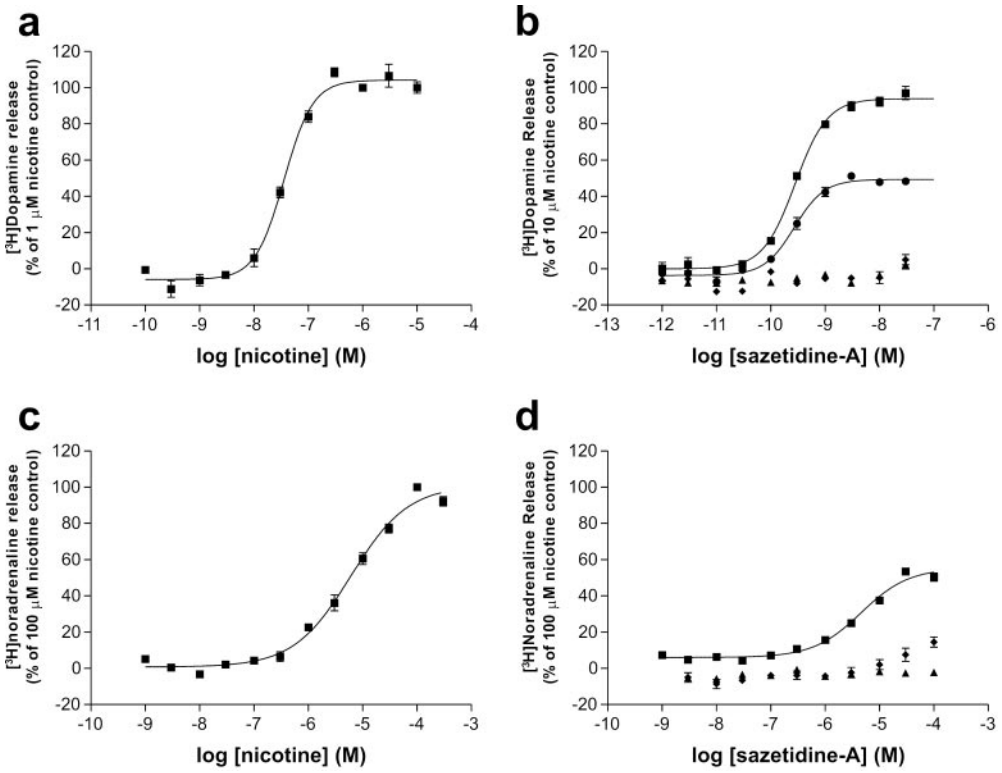

Fig. 3. Nicotine and sazetidine-A-induced neurotransmitter release. a, nicotine evokes dopamine release from rat striatal slices in a concentration-dependent manner (EC_{50} = 36 ± 8 nM; E_{max} 104 ± 3%; n = 3). b, sazetidine-A evokes dopamine release from rat striatal slices in a concentration-dependent manner (■). Sazetidine-A-evoked dopamine release is prevented in the presence of the nicotinic receptor antagonists dihydro-β-erythroidine (100 μM; ♦) and mecamylamine (10 μM; ▲). α-Conotoxin MII (0.1 μM; ●) reduces the E_{max} of sazetidine-A by 48%, but does not affect its EC_{50}. c, nicotine evokes noradrenaline release from rat hippocampal slices in a concentration-dependent manner (EC_{50} = 6.0 ± 2.4 μM; E_{max} 101 ± 5%; n = 3). d, sazetidine-A evokes noradrenaline release from rat hippocampal slices in a concentration-dependent manner (■). Sazetidine-A evoked noradrenaline release is prevented in the presence of the nicotinic receptor antagonists dihydro-β-erythroidine (100 μM; ♦) and mecamylamine (10 μM; ▲).

nAChRs, reduced the E_{max} of the concentration-response curve of sazetidine-A by approximately 48%, indicating that approximately half of the sazetidine-A-induced dopamine release is mediated by α6 subunit-containing nAChRs. Thus, besides being an agonist of $\alpha4\beta2$* nAChRs, sazetidine-A is also a potent agonist of α6 subunit-containing nAChRs.

In particular, the agonistic effects on $\alpha4\beta2$* nAChRs were quite surprising, specifically because in SH-EP1 cells stably transfected with recombinant human $\alpha4\beta2$ nAChRs, sazetidine-A does not induce any measurable agonistic effect (Xiao et al., 2006). In contrast, prolonged (but not acute) applications of low concentrations of sazetidine-A potently desensitized $\alpha4\beta2$ nAChRs expressed in the same cells, an effect more typically associated with agonists. This led the authors to suggest that sazetidine-A is a "silent desensitizer" of $\alpha4\beta2$ nAChRs (Xiao et al., 2006).

To identify the molecular basis for this apparent discrepancy, we performed more electrophysiological experiments. In the *X. laevis* oocyte expression system the expression of $\alpha4\beta2$ nAChRs subtypes with high- and low-sensitivity to ACh can be manipulated by transfecting the oocytes with cDNAs encoding the $\alpha4$ and $\beta2$ subunits in different ratios (Zwart and Vijverberg, 1998; Moroni et al., 2006; Zwart et al., 2006). When $\alpha4$ and $\beta2$ subunits are injected in the 1:10 $\alpha4/\beta2$ ratio, the oocytes will express a homogeneous population of $\alpha4\beta2$ nAChRs with high sensitivity to ACh, whereas injection of $\alpha4$ and $\beta2$ subunits in the 10:1 ratio results in the expression of a homogeneous population of $\alpha4\beta2$ nAChRs with low sensitivity to ACh. When sazetidine-A was tested on both $\alpha4\beta2$ nAChR subtypes, it was found to be a potent and efficacious agonist of the $\alpha4\beta2$ nAChRs that have high sensitivity to ACh. It must be stressed, however, that sazetidine-A also potently activated the second isoform of $\alpha4\beta2$ nAChRs with low sensitivity to ACh but with very low efficacy.

The question then arises as to why Xiao et al. (2006) observed no agonist effect of sazetidine-A on their heterologously expressed $\alpha4\beta2$ nAChRs. First of all, the fact that recombinant $\alpha4$ and $\beta2$ nAChR subunits assemble in more than one $\alpha4\beta2$ nAChR stoichiometry was totally neglected, and the subtypes of $\alpha4\beta2$ nAChRs expressed in SH-EP1 cells were not verified. This is an important factor, because potencies and efficacies of $\alpha4\beta2$ nAChR ligands depend heavily on the subunit stoichiometry of $\alpha4\beta2$ nAChRs (Zwart and Vijverberg, 1998; Nelson et al., 2003; Zhou et al., 2003; Karadsheh et al., 2004; Khiroug et al., 2004; López-Hernández et al., 2004; Briggs et al., 2006; Moroni et al., 2006; Zwart et al., 2006; Tapia et al., 2007). One possibility is that the SH-EP1 cells used by Xiao et al. (2006) express mainly $\alpha4(3)\beta2(2)$ nAChRs that have low affinity for ACh. In combination with the poorly sensitive biochemical rubidium efflux assay used, the very partial agonist effect of sazetidine-A on the low-

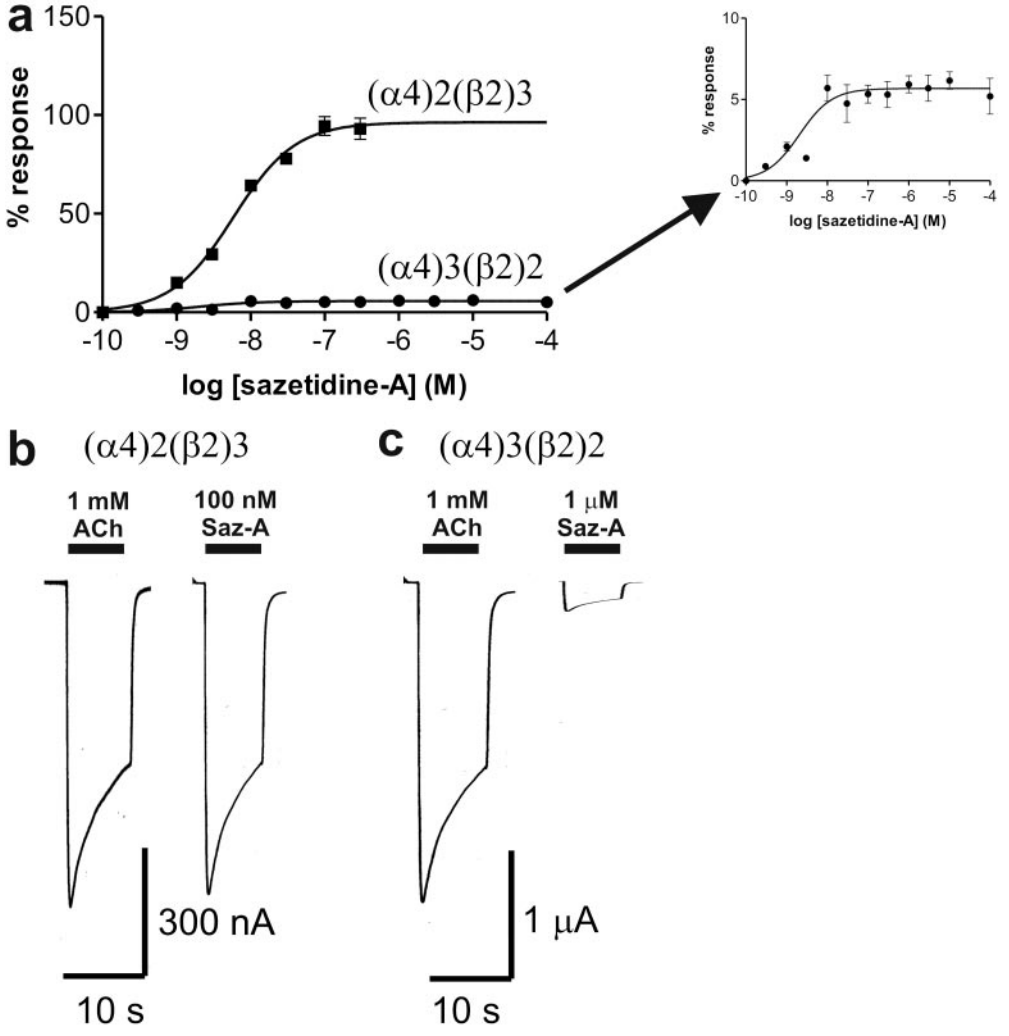

Fig. 4. Agonist effects of sazetidine-A on oocytes heterologously expressing $\alpha4\beta2$ nAChRs that have high sensitivity to ACh ($\alpha4(2)\beta2(3)$) and $\alpha4\beta2$ nAChRs that have low sensitivity to ACh ($\alpha4(3)\beta2(2)$). a, concentration-response curves of sazetidine-A to evoke ion currents in oocytes expressing $\alpha4(2)\beta2(3)$ nAChRs (■) and in oocytes expressing $\alpha4(3)\beta2(2)$ nAChRs (●). The inset shows the agonist concentration-response curve of sazetidine-A on $\alpha4(3)\beta2(2)$ nAChRs, showing that sazetidine-A is a potent but partial agonist on this subtype of $\alpha4\beta2$ nAChR. B, ion currents evoked by maximum-effective concentrations of ACh and sazetidine-A in oocytes expressing $\alpha4(2)\beta2(3)$ nAChRs, showing that sazetidine-A is a full agonist on this subtype of $\alpha4\beta2$ nAChR. C, ion currents evoked by maximum-effective concentrations of ACh and sazetidine-A in oocytes expressing $\alpha4(3)\beta2(2)$ nAChRs, showing that sazetidine-A is a partial agonist on this subtype of $\alpha4\beta2$ nAChR.

affinity subtype of $\alpha4\beta2$ nAChR might have been missed. However, two reports from another laboratory show concentration-response curves for ACh in the same human $\alpha4\beta2$ SH-EP1 cell line using a rubidium efflux assay with EC_{50} values of approximately 1 μM (Eaton et al., 2003; Kuo et al., 2005), suggesting that these cells might express $\alpha4(2)\beta2(3)$ nAChRs that have high affinity for ACh. A patch clamp study on the same cells revealed an intermediate EC_{50} for ACh of approximately 10 μM and a shallow slope factor of 0.6, suggesting that these cells express a mixture of $\alpha4\beta2$ nAChRs with high- and low-sensitivity for ACh (Wu et al., 2004). Taken together, the data seem to indicate that different labs find different pharmacological properties with the same cell line. An explanation for this could be that cell culture conditions vary between labs. It is known that the proportion of $\alpha4\beta2$ nAChRs with high- and low-affinity for ACh depends on cell culture conditions (e.g., temperature during incubation of the cells) (Nelson et al., 2003; Zwart et al., 2006). Along these lines, it is possible that the major difference between the present findings on $\alpha4\beta2$ nAChRs expressed in *X. laevis* oocytes and the previously published data on this compound (Xiao et al., 2006) in $\alpha4\beta2$ nAChRs expressed in a mammalian cell line might be due to differences in the expression system used.

Our results help to understand and reconcile the recent reports on in vivo effects of sazetidine-A, showing that it acts as a quite typical $\alpha4\beta2$ nAChR agonist in both drug discrimination (Xiao et al., 2007) and analgesic (Cucchiaro et al., 2007) assays. Although the contribution of additional subunits in native $\alpha4\beta2$* nAChRs has not been excluded, the results are consistent with the hypothesis that in vivo effects of $\alpha4\beta2$ nAChR agonists are mediated by native a4b2* nAChRs that have an $\alpha4(2)\beta2(3)$ stoichiometry.

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